Impaired Glial Glutamate Uptake Induces Extrasynaptic Glutamate Spillover in the Spinal Sensory Synapses of Neuropathic Rats

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Nie H, Weng HR. Impaired glial glutamate uptake induces extrasynaptic glutamate spillover in the spinal sensory synapses of neuropathic rats. J Neurophysiol 103: 2570–2580, 2010. First published March 10, 2010; doi:10.1152/jn.00013.2010. Glial cell dysfunction and excessive glutamate receptor activation in spinal dorsal horn neurons are hallmark mechanisms of pathological pain. The way in which glial cell dysfunction leads to excessive glutamate receptor activation in the spinal sensory synapses remains unknown. We and others recently reported the downregulation of glial glutamate transporter (GT) protein expression in the spinal dorsal horn of neuropathic rats. In this study, we showed that excitatory postsynaptic currents originating from N-methyl-D-aspartate receptor activation (NMDA EPSCs) elicited by peripheral synaptic input in the spinal sensory synapses were enhanced in neuropathic rats with mechanical allodynia induced by partial sciatic nerve ligation. The enhanced NMDA EPSCs were accompanied by an increased proportion of NR2B receptor activation. Physically blocking the extrasynaptic glutamate with dextran or chemically scavenging the gluta- matic-pyruvic transaminase ameliorated the abnormal NMDA EPSCs in neuropathic rats. Pharmacological blockade of glial GTs with dihydrokainic acid enhanced NMDA receptor activation elicited by synaptic input or puffed glutamate in normal control rats, but this effect was precluded in neuropathic rats. Thus extrasynaptic glutamate spillover and excessive activation of NMDA receptors resulting from deficient glutamate uptake in the synapses resulted in the excessive activation of NMDA receptors in neuropathic rats. It is suggested that extrasynaptic gluta- matic spillover may be a key synaptic mechanism related to phenotypic alterations induced by nerve injury in the spinal dorsal horn and that glial GTs are potential new targets in the development of analgesics.

INTRODUCTION

Dysfunction of glial cells and excessive activation of glutamate receptors in spinal dorsal horn neurons are hallmark mechanisms of pathological pain (Milligan and Watkins 2009; Moore et al. 2000; Ren and Dubner 2008). Activation of glutamate receptors is governed by three essential factors: the amount of synaptically released glutamate, the rate at which glutamate is removed by glutamate transporters (GTs), and the properties of postsynaptic glutamate receptors (Anderson and Swanson 2000; Clements 1996). Researchers have gone to great efforts to understand mechanisms related to the pronociceptive effects of glial inflammatory cytokines (Milligan and Watkins 2009), glutamate release from presynaptic terminals (Campbell and Meyer 2006; Carlton and Neugebauer 2002; Woolf and Ma 2007), and activation of glutamate receptors (Kalila et al. 2008) and their intracellular signal pathways in postsynaptic neurons (Ji and Strichartz 2004). However, how dysfunction of glial cells results in abnormal activation of glutamate receptors in the spinal sensory synapses in pathological pain remains to be established.

Glutamate released from presynaptic neurons is not metabolized extracellularly; rather clearance of glutamate in the synaptic cleft and homeostasis of extracellular glutamate are ensured by a family of GTs located in the plasma membranes of both glial cells and neurons that uptake glutamate from the extracellular space into the cells (Danbolt 2001; Jonas 2000; Trussell 1998). Uptake of glutamate by GTs is driven by the electrochemical gradients of Na⁺ and K⁺ across the plasma membrane (Danbolt 2001; Gegelashvili et al. 2001). Three types of GTs (glial GLAST and GLT-1 and neuronal EAAC1) exist in the spinal cord (Furuta et al. 1997; Mao et al. 2002; Seal and Amara 1999; Sung et al. 2003; Tao et al. 2004; Vera-Portocarrero et al. 2002; Weng et al. 2005). In general, glial GTs are believed to account for >90% of all synaptic glutamate uptake in the CNS (Danbolt 2001).

We and others recently investigated the role of GTs in the pain signaling system. Selective blockade of GTs in the spinal cord induced hypersensitivity to heat and mechanical stimulation (Liaw et al. 2005; Weng et al. 2006) and enhanced activation of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors at the synaptic level (Nie and Weng 2009; Weng et al. 2006, 2007). Our studies identified extrasynaptic glutamate spillover as a key mechanism of excessive activation of glutamate receptors resulting from deficient glutamate uptake in the spinal dorsal horn (Nie and Weng 2009; Weng et al. 2006, 2007). Hyperalgesia induced by chronic nerve injury (Sung et al. 2003; Xin et al. 2009), chemotherapy (e.g., paclitaxel) (Weng et al. 2005), or opioids (Mao et al. 2002; Thomson et al. 2006) is associated with downregulation of GT protein expression in the spinal dorsal horn. However, direct evidence demonstrat- ing the impact of downregulation of glial GT protein expression on activation of ionotropic glutamate receptors in the spinal sensory synapses of neuropathic rats is still lacking.

In this study, we demonstrated that deficient glutamate uptake by glial cells in the spinal sensory synapses resulted in extrasynaptic glutamate spillover and activation of extrasynaptic NMDA receptors, which is a culprit causing enhanced NMDA receptor activation elicited by peripheral afferent inputs in neuropathic rats induced by partial sciatic nerve ligation (pSNL).

METHODOLOGY

Animals

Young adult male Sprague-Dawley rats (weight range, 150–220 g) were used. All animal experiments were approved by The University.
of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee and were fully compliant with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

pSNL and behavioral tests

Under isoflurane-induced (2–3%) anesthesia, the left sciatic nerve at the upper thigh was exposed and tightly ligated with 5×0 silk sutures to approximately one-third to one-half the thickness of the sciatic nerve as described by Seltzer et al. (1990). The wound was closed with muscle sutures and skin staples. In sham-operated rats, the left sciatic nerve was exposed but not ligated. To verify the development of tactile allodynia after pSNL, behavioral tests were performed to measure the mechanical sensitivity of both hind paws prior to and after surgery (Weng et al. 2003). Briefly, the animals were placed on wire mesh, loosely restrained under a Plexiglass cage (12 × 20 × 15 cm), and allowed to accommodate for ≥15 min. Von Frey monofilaments with bending forces ranging from 0.1 to 8.0 g were applied from below through the mesh onto the mid-plantar area of each hind paw to evoke paw withdrawal responses. Each hind paw was stimulated 10 times with each Von Frey monofilament, and the frequency (percentage) of paw withdrawal responses to 10 stimulations was recorded (Weng et al. 2003). The least bending force that evoked withdrawal in more than half the trials was assigned as the 50% withdrawal threshold (Cata et al. 2004; Weng et al. 2003).

In vitro whole cell recordings

SPINAL SLICE PREPARATION. Rats were deeply anesthetized via intraperitoneal injection of urethane and underwent laminectomy for removal of the lumbar spinal cord. The lumbar spinal cord section was placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF) saturated with 95% O2–5% CO2. The sucrose ACSF contained (in mM) 24 sucrose, 3.6 KCl, 1.2 MgCl2, 2.5 CaCl2, 1.2 NaH2PO4, 12.0 glucose, and 25.0 NaHCO3. The pia-arachnoid membrane was removed from the section. The L4–5 spinal segment, identified by the lumbar ice-cold sucrose artificial cerebrospinal fluid (ACSF) presaturated lumbar spinal cord. The lumbar spinal cord section was placed in 0.80 g).

RESULTS

All of the pSNL rats had mechanical allodynia prior to undergoing the electrophysiological experiments. The mechanical threshold ipsilateral to the pSNL side significantly decreased from 5.64 ± 0.4 g at baseline to 1.85 ± 0.32 g (P < 0.001) prior to the electrophysiological recordings in 28 nerve-ligated rats. The mechanical threshold in five sham-operated rats was not significantly altered (from 5.60 ± 0.32 g to 4.20 ± 0.80 g).

We recorded NMDA EPSCs from the neurons in the spinal superficial dorsal horn (laminae I and II) in the L4 and L5 segments. To evaluate the activation of NMDA receptors in the synapses responding to weak primary input and synapses transmitting strong primary input, we evoked NMDA EPSCs using electrical stimulation of the spinal dorsal root entry zone at two stimulating intensities: one at twice the EPSC activation threshold (2T) and another at the intensity that evokes a maximum EPSC in the recorded neuron (maximum stimulation) (Nie and Weng 2009). The electrophysiological recordings were taken from the 28 nerve-ligated rats 8–14 days after an Axopatch 700B (Molecular Devices) and displayed and stored in a personal computer.

Excitatory postsynaptic currents (EPSCs) were evoked using constant-current electrical stimuli (0.2-ms duration repeated every 45 s) applied with a concentric bipolar stimulating electrode placed at the dorsal root entry zone (Weng et al. 2006; Yoshimura and Nishi 1993). For each cell, AMPA EPSCs were first evoked at a holding potential of −70 mV by electrical stimulation of the spinal dorsal root entry zone. NMDA receptor currents were then isolated by including 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM), bicuculline (10 μM), and strychnine (5 μM) in the external solution to block non-NMDA glutamate (AMPA and kainate) receptors, GABA A, and glycine receptors and holding the membrane potential at +40 mV to remove the voltage-dependent Mg2+ block from NMDA receptors. In a subset of experiments, NMDA currents evoked by exogenous L-glutamate (glu-NMDA current) and those evoked by exogenous NMDA (agonist-NMDA current) were recorded from the same neuron in the presence of tetrodotoxin (TTX). This recording was achieved by superfusing L-glutamate (50 μM, 20-ms duration) and NMDA (200 μM, 20-ms duration), respectively, onto the recording cells through a double-barrel pipette with an opening tip size of 8–12 μm.

MATERIALS. DNQX, bicuculline, strychnine, L-glutamate, NMDA, dextran, dihydrokainic acid (DHK), glutamic-pyruvic transaminase, and TTX were obtained from Sigma, and TBOA, Ro 25–6981, and 2-amino-5-phosphonovaleric acid (AP5) were obtained from Tocris Bioscience. All pharmacological agents were applied via perfusion into the recording chamber.

DATA ANALYSIS. Data were recorded using Axopatch 700B amplifiers, digitized at 10 kHz, and analyzed off-line. The mean of three to four EPSCs evoked by electrical stimulation or by superfused L-glutamate or NMDA at baseline and in the presence of tested drugs was measured. To determine time constants for the decay phase of NMDA EPSCs, the decay phase was fitted with a monoeponential function (Weng et al. 2007). The Clampfit software program (version 10.2; Molecular Devices) was used to detect and measure the peak latency (time from stimulation onset to peak), amplitude, duration, area, and time constant of averaged EPSCs. The data were presented as the means ± SE. Student’s t-test was used to determine statistical differences between data obtained before and after tested drugs (paired t-test) or between groups (nonpaired t-test). Fisher’s test was used to compare incidence rates between groups. A P value <0.05 was considered statistically significant.

Throughout the experiment, the animals were given 50% O2–5% CO2 at 35°C for the dorsal root entry zone (Weng et al. 2006; Yoshimura and Nishi 1993). For each cell, AMPA EPSCs were first evoked at a holding potential of −70 mV by electrical stimulation of the spinal dorsal root entry zone. NMDA receptor currents were then isolated by including 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM), bicuculline (10 μM), and strychnine (5 μM) in the external solution to block non-NMDA glutamate (AMPA and kainate) receptors, GABA A, and glycine receptors and holding the membrane potential at +40 mV to remove the voltage-dependent Mg2+ block from NMDA receptors. In a subset of experiments, NMDA currents evoked by exogenous L-glutamate (glu-NMDA current) and those evoked by exogenous NMDA (agonist-NMDA current) were recorded from the same neuron in the presence of tetrodotoxin (TTX). This recording was achieved by superfusing L-glutamate (50 μM, 20-ms duration) and NMDA (200 μM, 20-ms duration), respectively, onto the recording cells through a double-barrel pipette with an opening tip size of 8–12 μm.

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surgery. We previously reported that during this period the protein expression of glial GTs (GLAST and GLT-1) in the spinal dorsal horn ipsilateral to the operated side was downregulated by 30–50% in the pSNL rats but not in the sham-operated rats (Xin et al. 2009). For comparison, electrophysiological data collected during the same period from 5 sham-operated rats and 27 normal unoperated rats were combined together as the normal control. The NMDA EPSC amplitude (82.43 ± 12.76 pA) and duration (517.15 ± 50.85 ms) evoked by 2T stimulation in 12 neurons from sham-operated rats were similar to those obtained from 39 neurons from the unoperated rats (amplitude: 76.73 ± 11.85 pA; duration: 610.74 ± 53.77 ms). Similarly, no significant differences were found between the sham-operated rats (12 neurons) and unoperated rats (39 neurons) in amplitude (sham: 231.09 ± 66.18 pA; unoperated: 183.37 ± 23.07 pA) and duration (sham: 906.59 ± 103.79 ms; unoperated: 1043.83 ± 69.39 ms) evoked by maximum stimulation.

Enhanced activation of NMDA receptors in spinal superficial dorsal horn neurons was associated with behavioral hypersensitivity induced by pSNL

We observed no significant differences in the NMDA EPSC activation threshold between the control (125.19 ± 18.49 μA; n = 51) and neuropathic (127.37 ± 14.84 μA; n = 39) rats. In comparison with those in control rats (51 neurons), NMDA EPSCs evoked by weak (2T) stimulation in neuropathic rats (39 neurons) had delayed peak latencies (27.73 ± 2.52 vs. 18.93 ± 1.19 ms; P < 0.01) and longer durations (1,068.86 ± 51.49 vs. 588.72 ± 42.96 ms; P < 0.001) and decay time constants (204.49 ± 20.26 vs. 144.91 ± 12.38 ms; P < 0.01). Similarly, the peak latency (27.45 ± 2.44 ms), duration (2,366.47 ± 189.85 ms), and decay time constant (259.37 ± 19.87 ms) of NMDA EPSCs evoked by maximum stimulation in neuropathic rats (39 neurons) were also significantly longer than those in control rats (51 neurons; peak latency: 19.61 ± 1.30 ms, P < 0.01; duration: 1,011.53 ± 58.51 ms, P < 0.001; decay time constant: 188.14 ± 11.99 ms, P < 0.01; Fig. 1). Although the mean EPSC amplitudes in control and neuropathic rats did not differ significantly, a greater proportion of neurons in neuropathic rats (84.21% of 39 neurons) than in normal rats (64.71% of 51 neurons; P < 0.05) had an EPSC amplitude >100 pA in response to maximum stimulation. Further analysis showed that the areas of NMDA EPSCs evoked by 2T and maximum stimulation in neuropathic rats were significantly larger than those found control rats (P < 0.01; Fig. 1). These data indicated that enhanced activation of NMDA receptors is associated with development of mechanical hypersensitivity in neuropathic rats and downregulation of glial GTs in the spinal dorsal horn (Xin et al. 2009). In addition, the delayed NMDA EPSC peak latencies, prolonged durations, and slow decay time constants further suggested increased diffusion distances between the sites of glutamate release and the target receptors or increased glutamate dwelling time after the glutamate release.

Increased proportion of NR2B receptor activation in NMDA EPSCs in spinal superficial dorsal horn neurons of neuropathic rats

Studies in forebrain areas (Diamond 2002) and our recently published findings on the spinal superficial dorsal horn (Nie and Weng 2009; Weng et al. 2007) showed that key features induced by deficient glutamate uptake in synapses are extrasynaptic glutamate spillover, activation of extrasynaptic NMDA receptors, and increased receptor activation in the presence of extrasynaptic glutamate. In this study, we sought to determine whether there is an increase in extrasynaptic NMDA receptor activation in neuropathic rats.

**Fig. 1.** Enhanced activation of N-methyl-D-aspartate (NMDA) receptors in spinal superficial dorsal horn neurons in neuropathic rats. A: samples of NMDA excitatory postsynaptic currents (NMDA EPSCs) evoked by 2T and maximum stimulation in control and neuropathic rats. B: bar graphs showing the mean ± SE amplitudes, peak latencies, durations, decay time constants, and areas of EPSCs. *Comparisons of EPSCs in control and neuropathic rats. **P < 0.01; ***P < 0.001.**
receptors, and an increased proportion of NR2B receptor activation in NMDA EPSCs. If impaired glutamate uptake contributes to enhanced activation of NMDA receptors in neuropathic rats, one should see extrasynaptic glutamate spillover and increased activation of NR2B receptors. We tested this first by comparing the effect of inhibition of NR2B subunits on the NMDA EPSCs in control and neuropathic rats. Selective inhibition of NR2B receptors was achieved by bath-application of a selective NR2B receptor antagonist, Ro 25–6981 (1 μM) (Fischer et al. 1997; Mutel et al. 1998). Ro 25–6981 significantly reduced the NMDA EPSC amplitude, duration, and decay time constant induced by both 2T and maximum stimulation in neuropathic rats (with P values ranging from 0.02 to <0.001). The percentage reductions (52.79 ± 3.54%; n = 6) induced by Ro 25–6981 in NMDA EPSC amplitudes elicited by maximum stimulation in neuropathic rats were significantly stronger than those (39.51 ± 5.38%; n = 7, P < 0.05) in control rats. The Ro 25–6981 induced reductions in NMDA EPSC durations elicited by 2T (39.62 ± 12.34%; n = 6) and maximum (49.72 ± 3.68%; n = 6) stimulation in neuropathic rats were significantly stronger than those in control rats (2T stimulation: 10.64 ± 4.21%, n = 7, P < 0.05; maximum stimulation: 18.02 ± 5.92%, n = 7, P < 0.01). Ro 25–6981 also produced significantly stronger reduction of the NMDA EPSC decay time constant induced by 2T (P < 0.05) or maximum stimulation (P < 0.01) in neuropathic rats (6 neurons) than control rats (7 neurons; Fig. 2). These data supported our assumption that an increased proportion of NR2B receptor activation in NMDA EPSCs contributes to prolonged activation of NMDA receptors in neuropathic rats. Alternatively, these alterations may result from increased NR2B receptor expression and/or altered kinetics of NR2B receptors inside the synapses after nerve injury.

**Limiting the spatial diffusion (spillover) of extrasynaptic glutamate ameliorated abnormal activation of NMDA receptors in neuropathic rats**

We next sought to directly determine the contribution of extrasynaptic activation to enhanced activation of NMDA receptors in neuropathic rats by limiting extrasynaptic glutamate spillover. Limiting this spillover should only reduce the number of NMDA receptors activated by abnormal extrasynaptic glutamate diffusion; it should not ameliorate abnormal activation of NMDA receptors induced by altered NMDA receptor properties inside the synapse. We first used dextran, a 40-kDa inert macromolecule, to prevent diffusion of glutamate in the extracellular space (ECS) (Lambe and Aghajanian 2006; Min et al. 1998). When dextran molecules are added to the ECS, they act as obstacles to the movement of diffusing glutamate, resulting in an increase in viscosity and a decrease in extrasynaptic glutamate diffusion (spillover) in the ECS. We examined the effect of dextran on NMDA EPSCs in the spinal superficial dorsal horn neurons of control and neuropathic rats by adding dextran in the perfusion solution (concentration: 5%) after recording responses at baseline. The increase in osmolarity by dextran was compensated by adding 6% of distilled water (Tsvetkov et al. 2004). In agreement with results of previous studies (Min et al. 1998; Tsvetkov et al. 2004) in the forebrain area, we found that the dextran treatment had no significant effect on the NMDA EPSCs in the spinal sensory synapses (tested in 8 neurons) in control rats (Fig. 3), indicating that increasing ECS viscosity does not change the glutamate transient inside the synaptic cleft and that extrasynaptic glutamate diffusion is minimal in control animals. In contrast, dextran significantly shortened the 2T stimulation-evoked NMDA EPSC peak latency, duration, and decay time constant by 17.09 ± 5.75% (P < 0.05), 24.44 ± 7.74% (P < 0.05), and 24.65 ± 5.93% (P < 0.05), respectively, in neuropathic rats (tested in 8 neurons; Fig. 3). Similarly, dextran significantly reduced the NMDA EPSC peak latency, duration, and decay time constant evoked by maximum stimulation by 25.40 ± 3.41% (P < 0.001), 39.52 ± 7.05% (P < 0.01), and 14.87 ± 5.76% (P < 0.05), respectively, in neuropathic rats. The decrease in glutamate diffusion induced by dextran was accompanied by a significant increase (17.58 ± 6.45%; P < 0.05) in the NMDA amplitude evoked by maximum stimulation (Fig. 3), indicating that more synaptic NMDA receptors were activated by glutamate accumulated inside the synaptic cleft as a result of less glutamate escaping to the extrasynaptic space. These data indicated that extrasynaptic glutamate diffusion (spillover) accounted for the delay peak latency and prolonged activation of NMDA receptors.

Another way to limit extrasynaptic glutamate spillover is to use a glutamate scavenger such as glutamic-pyruvic transaminase, which rapidly metabolizes extrasynaptic glutamate in the presence of pyruvate without modifying glutamate transients in the synaptic cleft (Min et al. 1998; Overstreet et al. 1999; Rossi et al. 2002; Tsvetkov et al. 2004). We found that adding glutamic-pyruvic transaminase (5 U/ml) to the perfusing solution significantly reduced the activation of NMDA receptors evoked by 2T and maximum stimulation in the superficial spinal dorsal horn neurons of neuropathic rats.
Impaired glial glutamate uptake resulted in prolonged activation of NMDA receptors by exogenous L-glutamate but not NMDA in neuropathic rats

The extrasynaptic glutamate spillover in neuropathic rats described in the preceding text may have resulted from impairment of glutamate uptake and/or increased release of glutamate from presynaptic terminals. To demonstrate that impairment of glutamate uptake by glial GTs is key to enhanced activation of NMDA receptors in neuropathic rats, we studied NMDA currents evoked by exogenous L-glutamate and NMDA in individual superficial dorsal horn neurons in neuropathic and control rats. These experiments were based on the substrate selectivity of GTs. GTs selectively uptake L-glutamate but not NMDA because the former is the substrate of GTs but the latter is not (Danbolt 2001; Jabaudon et al. 1999). In other words, NMDA currents evoked by exogenous L-glutamate (glu-NMDA current) are regulated by the functional status of GTs, but NMDA currents evoked by exogenous NMDA (agonist-NMDA current) are not. We first confirmed this principle in spinal slices obtained from control rats. We evoked glu-NMDA current and agonist-NMDA current with similar amplitudes (~80–100 pA) in the same neuron by puffing L-glutamate (50 μM, 20-ms duration) and NMDA (200 μM, 20-ms duration) during maximum stimulation in spinal superficial dorsal horn neurons in control rats. We confirmed that this scavenger does not change the glutamate receptor activation are key to enhanced activation of glutamate receptors in neuropathic rats.

**FIG. 4.** Limiting extrasynaptic glutamate spillover with the glutamate scavenger glutamic-pyruvic transaminase reduced the NMDA EPSC peak amplitude, latency, duration, and decay time constant in neuropathic rats but not in control rats. A: original recordings showing samples of NMDA EPSCs evoked by maximum stimulation in spinal superficial dorsal horn neurons in control (left) and neuropathic (right) rats recorded before (top) and during (middle) perfusion of 5% dextran. Bottom: overlaps of the top and middle. B: bar graphs showing the mean percentage changes in the NMDA EPSC amplitude, peak latency, duration, and decay time constant induced by dextran. *Comparisons of the effects induced by dextran on NMDA EPSCs in control and neuropathic rats. *P < 0.05; ***P < 0.001.
μM, 20-ms duration) (Ohno-Shosaku et al. 2007; Zhao et al. 2008), respectively, onto the recorded cell through a double-barrel pipette. Although the puff duration was kept constant at 20 ms throughout the whole project, the puff air pressure (3–6 psi) for each barrel was optimized so that the amplitudes of NMDA currents evoked by puffing l-glutamate and NMDA were similar (glu-NMDA current, 89.95 ± 6.01 pA; agonist-NMDA current, 92.23 ± 5.53 pA, n = 6). The agonist-NMDA current evoked by puffing NMDA in six neurons had a mean peak latency of 290.30 ± 65.24 ms, duration of 5,510.04 ± 892.53 ms, and decay time constant of 1,596.19 ms. These were significantly longer than those evoked by puffing l-glutamate (peak latency, 70.98 ± 11.87 ms, P = 0.01; duration, 535.84 ± 116.35 ms, P < 0.01; decay time constant, 117.89 ± 26.49 ms P < 0.01; Fig. 5, A and B). Currents evoked by puffing l-glutamate or NMDA were completely inhibited by perfusing n-AP5 (25 μM; n = 4) into the recording bath, confirming that the recorded currents were caused by activation of NMDA receptors. The differences between the glu-NMDA current and agonist-NMDA current indicated that l-glutamate stayed in the ECS for a shorter period than NMDA did even though the effects of puffing NMDA and of puffing l-glutamate on the number of NMDA channels activated (i.e., the amplitude of NMDA currents) were similar. Furthermore, bath perfusion of a selective glial GT blocker DHK (300 μM, tested in 6 neurons) (Arriza et al. 1994; Zhang et al. 2009) significantly increased the glu-NMDA current amplitude (from 89.95 ± 6.49 to 162.22 ± 29.83 pA; P < 0.05), peak latency (from 70.98 ± 11.87 to 123.87 ± 23.79 ms; P < 0.01), duration (from 535.84 ± 116.35 to 2015.58 ± 526.93 ms; P = 0.01), and decay time constant (from 117.89 ± 26.49 to 353.9 ± 90.47 ms; P < 0.01). In contrast, bath perfusion of 300 μM DHK did not alter agonist-NMDA currents (n = 6; Fig. 5, A and C). These data confirmed that glial GTs regulate activation of NMDA receptors by l-glutamate but not activation of NMDA receptors by NMDA.

Using the same approach, we then evoked glu-NMDA current and agonist-NMDA current in the same neuron in neuropathic rats. For comparisons with control rats, NMDA current amplitudes evoked in neuropathic rats were similar to those in control rats (Fig. 5, A and B). These were achieved by adjusting the puff air pressure (3–6 psi). In comparison with glu-NMDA current in control rats (6 neurons), glu-NMDA current evoked by 20-ms puffing l-glutamate in neuropathic rats (8 neurons) had a significantly longer mean peak latency (1,77.71 ± 37.24 vs. 70.98 ± 11.87 ms; P = 0.01), duration (2,534.48 ± 458.69 vs. 535.84 ± 116.35 ms; P = 0.002), and decay time constant (845.53 ± 226.4 vs. 117.89 ± 26.49 ms; P < 0.01; Fig. 5, A and B). Furthermore, at similar agonist-NMDA current amplitudes evoked by 20-ms puffing NMDA in control (92.23 ± 5.98 pA; n = 6) and neuropathic (101.58 ± 7.84 pA; n = 8) rats, we observed no significant differences in the agonist-NMDA current peak latency, duration, or decay time constant between control and neuropathic rats. Currents

**FIG. 5.** Impairment of glutamate clearance by glial glutamate transporters (GTs) in neuropathic rats is key to prolonged activation of NMDA receptors evoked by exogenous glutamate in neuropathic rats. A: original recordings showing NMDA currents evoked by puff application (20 ms) of l-glutamate (glu-NMDA current) and NMDA (agonist-NMDA current) recorded, respectively, in the same neuron at baseline and during perfusion of dihydrokainic acid (DHK, 300 μM) in control and neuropathic rats. † onset of puff application. The recordings were obtained for spinal superficial dorsal horn neurons in the presence of TTX (1 μM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM) at a holding potential of +40 mV. B: mean amplitudes, peak latencies, durations, and decay time constants for glu-NMDA current and agonist-NMDA current in control rats and neuropathic rats. C: mean percentage changes induced by DHK in glu-NMDA current and agonist-NMDA current in control and neuropathic rats. *Comparison between control and neuropathic rats. *P < 0.05. **P < 0.01. ***P < 0.001.
evoked by puffing l-glutamate or NMDA in neuropathic rats were completely inhibited by n-AP5 (25 μM; \( n = 5 \)). These data indicated that alterations in the NMDA receptor properties or subunit composition are not primary contributors to prolonged activation of NMDA receptors activated by l-glutamate in neuropathic rats. Rather they demonstrated that impaired glutamate clearance by GTs is key to prolonged activation of NMDA receptors in neuropathic rats.

GTs are located in both glial cells and neurons in the spinal dorsal horn (Sung et al. 2003; Weng et al. 2005; Xin et al. 2009). If impairment of glutamate uptake by glial GTs is a culprit causing the abnormal spatiotemporal profile of presynaptic glutamate concentrations in neuropathic rats, the potentiation of glutamate-NMDA currents by blockade of glial GTs seen in control rats should be precluded or reduced in neuropathic rats because the effect of glial GT blockers is proportional to the number of functional glial GTs. As expected, selective blockade of glial GTs with 300 μM DHK in the bath did not significantly alter the amplitude, peak latency, duration, or decay time constant of glutamate-NMDA current evoked by 20-ms puffing of l-glutamate in neuropathic rats. In contrast, the same treatment of 300 μM DHK significantly increased the glutamate-NMDA current amplitude by 87.81 ± 39.88% (\( P < 0.05 \)), peak latency by 71.11 ± 10.19% (\( P < 0.01 \)), duration by 281.17 ± 71.74% (\( P = 0.01 \)), and decay time constant by 199.13 ± 41.21% (\( P < 0.01 \)) in control rats (Fig. 5, A and C). Similar to those found in control rats, DHK had no effects on the agonist-NMDA current peak latency, duration, or decay time constant in neuropathic rats (Fig. 5, A and C).

**Impaired glutamate uptake by glial GTs is key to the prolonged activation of NMDA receptors in the spinal synapses of neuropathic rats**

To further determine the impact of deficient glutamate uptake induced by glial cells on NMDA receptors specifically activated by peripheral synaptic input, we examined the effect of DHK on NMDA EPSCs evoked by stimulation of the spinal dorsal root entry zone in control and neuropathic rats. We found that NMDA EPSCs evoked by 2T and maximum stimulation in the spinal superficial dorsal horn neurons of control rats were significantly enhanced after perfusion of 300 μM DHK (Fig. 6). For example, DHK significantly increased the 2T-evoked NMDA EPSC amplitude by 69.36 ± 28.1% (\( P < 0.01 \)), peak latency by 57.51 ± 20.76% (\( P < 0.01 \)), duration by 77.19 ± 16.88% (\( P = 0.01 \)), and decay time constant by 23.37 ± 5.58% (\( P < 0.001 \)). However, this DHK-induced potentiation of NMDA EPSCs was almost completely precluded in neuropathic rats as the EPSC peak amplitude, latency and duration evoked by weak (2T) and maximum stimulation were not significantly altered after bath perfusion of 300 μM DHK. Thus the effects of DHK on the NMDA EPSC amplitude, peak latency, duration, and decay time constant in control rats were significantly greater (\( P \) values ranging from <0.05 to <0.01) than those in neuropathic rats (Fig. 6).

Taken together, we concluded that impaired glutamate uptake by glial GTs in the synapse is key to extrasynaptic glutamate spillover and activation of extrasynaptic NMDA receptors, which results in prolonged activation of NMDA receptors in spinal superficial dorsal horn neurons of neuropathic rats.

**Discussion**

Given mounting evidence that downregulation of glial GT protein expression in the spinal dorsal horn is associated with development of hyperalgesia and allodynia induced by peripheral nerve injury, elucidating the precise synaptic mechanisms by which this occurs bears important implications for the management and prevention of pathological pain. In this study, we demonstrated that deficient glutamate uptake by glial GTs leads to excessive activation of NMDA receptors in spinal sensory synapses in neuropathic rats. Specifically, we observed that mechanical allodynia induced by pSNL is associated with downregulation of expression of glial GT proteins reported previously (Xin et al. 2009) and enhanced activation of NMDA receptors in the spinal superficial dorsal horn. These are accompanied with an increased proportion of NR2B receptor activation. Limiting the spatial diffusion (spillover) of extrasynaptic glutamate with dextran or a glutamate scavenger ameliorates abnormal activation of NMDA receptors in neuropathic rats. Pharmacological blockade of glial GTs with dihydrokainic acid enhanced NMDA receptor activation elicited by synaptic input or puffed-glutamate in normal control rats, but not in neuropathic rats. These results indicate that extrasynaptic glutamate spillover induced by deficient glutamate uptake...
by glial GTs in the synapses results in excessive activation of NMDA receptors in neuropathic rats. Thus our study revealed a novel synaptic mechanism underlying abnormal neuronal transmission mediated by dysfunctional glial cells during the pathogenesis of pain.

The phenotype of dorsal horn neuron responses to peripheral stimulation in neuropathic animals includes increased response amplitudes, prolonged after discharges, and enlarged peripheral receptive field sizes (Bebehan and Dohler-Stolik 1994; Laird and Bennett 1993; Palecek et al. 1992; Weng et al. 2003). Multiple factors can contribute to these phenotypic alterations, including changes in the balance between presynaptic excitatory and inhibitory input, the active and passive electrophysiological membrane properties of the dorsal horn neurons, and properties of postsynaptic excitatory and inhibitory ligand-gated ion channels. In the present study, we identified increased activation of excitatory ligand-gated ion channels (NMDA receptors) as an important cause of increased activation of spinal dorsal horn neurons after nerve injury, which is consistent with previously reported data (Baba et al. 2000; Balasubramanyan et al. 2006; Iwata et al. 2007). Increased activation of excitatory ionotropic glutamate receptors in postsynaptic neurons can result from any of the following alone or combined: increased glutamate release from presynaptic neurons, decreased glutamate uptake by GTs, and increased numbers or conductance of postsynaptic glutamate receptors (Anderson and Swanson 2000; Clements 1996).

Our present study demonstrated that increased extrasynaptic glutamate concentrations (extrasynaptic glutamate spillover) were important contributors to enhanced activation of NMDA receptors in neuropathic rats. This agrees with previous reports demonstrating elevated extracellular glutamate concentrations in the spinal cord in animals with chronic constriction of the sciatic nerve (Sung et al. 2007) or subcutaneous injection of formalin (Malmborg et al. 2006). Increased release of presynaptic glutamate, as demonstrated by increased frequencies of miniature EPSCs in dorsal horn neurons (Balasubramanyan et al. 2006; Moore et al. 2002), may be attributed to dishomoeostasis of extracellular glutamate concentrations. Decreased glutamate uptake in the spinal cord (Binns et al. 2005; Sung et al. 2007) is another key factor leading to loss of glutamate homeostasis induced by peripheral nerve injury.

The importance of GTs in pain signaling has been demonstrated in behavioral and electrophysiological experiments. Deficiency in glutamate reuptake induced by selective GT inhibitors elevates spinal extracellular glutamate concentrations (Liaw et al. 2005) and produces spontaneous nociceptive behaviors and hypersensitivity to mechanical and thermal stimuli (Liaw et al. 2005; Weng et al. 2006). Spinal wide-dynamic range neurons exhibit increased spontaneous activity, innocuous and noxious stimulus-evoked responses, and after discharges following inhibition of GTs (Weng et al. 2006). The relationship of glutamate transporters with pathological pain has been further indicated by decreases in GT protein expression or glutamate uptake activity in the spinal dorsal horn in hyperalgesic rats induced by peripheral nerve injury (Binns et al. 2005; Sung et al. 2003, 2007), chemotherapy (Taxol) (Weng et al. 2005), and opioids (Mao et al. 2002; Thomson et al. 2006). However, it remains unknown whether such low levels of glial GT expression result in abnormal activation of glutamate receptors. In the present study, we showed that NMDA EPSCs evoked by peripheral input in neuropathic rats had delayed peak latencies, prolonged durations, and increased decay time constants. We found evidence that deficient glutamate uptake in the synapse by glial cells is a key contributor to such abnormal activation of NMDA receptors. These findings agree with recent studies by our group in which pharmacological blockade of GTs caused prolonged activation of AMPA and NMDA receptors and extrasynaptic glutamate spillover in spinal sensory synapses (Nie and Weng 2009; Weng et al. 2007). Amplitudes of NMDA EPSCs were significantly increased by pharmacological blockade of GTs (Nie and Weng 2009; Weng et al. 2007). However, the mean amplitudes of NMDA EPSCs in neuropathic rats were not significantly different from those in control rats, although we did find a greater proportion of neurons that had an EPSC amplitude >100 pA in response to maximum stimulation in neuropathic rats (84.21% of 39 neurons) than in control rats (64.71% of 51 neurons). The lack of difference between the mean amplitudes in neuropathic and control rats may have resulted from a wide range of NMDA EPSC amplitudes owing to the cutting of some primary afferent fibers projecting to the recorded neurons in the slice preparations.

Interestingly, while pSNL caused a 30–50% downregulation of glial GT protein expression in the spinal dorsal horn (Xin et al. 2009), the potentiation effect induced by 300 μM DHK on NMDA EPSCs seen in normal control rats was precluded in neuropathic rats. A simple explanation for this preclusion is that the glial GTs expressed in neuropathic rats do not functionally uptake glutamate. However, this does not likely underscore the core mechanisms leading to the preclusion of DHK effects, as the degree of reduction in glutamate uptake measured using spinal synaptosome preparations was reportedly comparable to the degree of downregulation of GT protein expression in the spinal cord induced by chronic constriction of the sciatic nerve (Sung et al. 2003). Instead it is likely that the degree of deficiency in glutamate uptake in neuropathic rats already produces a ceiling effect on the number of NMDA receptors activated by glutamate released by presynaptic terminals or exogenous puff-application prior to application of DHK.

Neurons in the spinal superficial dorsal horn receive nociceptive inputs from Aδ and C fiber primary afferents (Kumazawa and Perl 1977, 1978). Types of neurons in this area include interneurons and spinalthalamic tract neurons. Although we may have recorded inhibitory interneurons in our study, it is generally accepted that the majority of neurons in this area are excitatory (Santos et al. 2007; Yang et al. 1998). More importantly, the enhanced activation of NMDA receptors we observed in this study was associated with hyperalgesic behavior induced by pSNL. It is tempting to speculate our current findings as a synaptic mechanism underlying the phenotypic alterations aforementioned in intact neuropathic animals (Bebehan and Dohler-Stolik 1994; Laird and Bennett 1993; Palecek et al. 1992; Weng et al. 2003). Fast synaptic transmission in the spinal dorsal horn is conducted mainly by activation of AMPA and NMDA receptors. Prolonged activation of NMDA receptors in dorsal horn neurons of neuropathic rats found in the current study may underlie or at least contribute to prolonged after discharges of action potentials in the dorsal horn neurons in intact neuropathic animals. Activation of extrasynaptic glutamate receptors by extrasynaptic glutamate...
Glial cells also release many other bioactive substances, such as proinflammatory cytokines, nitric oxide, ATP, and prostaglandins, in response to peripheral tissue inflammation or injury (Watkins et al. 2001). Recent studies showed that proinflammatory cytokines can enhance activation of AMPA and NMDA receptors but decrease activation of GABA and glycine receptors in spinal lamina II neurons (Kawasaki et al. 2008). However, because the researchers in that study used agonists (AMPA and NMDA, neither of which are substrates of GTs) to activate AMPA and NMDA receptors, the role of glial GTs in abnormal synaptic transmission during the pathogenesis of pain remains unknown. In the present study, we identified deficient glutamate uptake as a novel mechanism for dysfunctional glial cells to enhanced activation of glutamate receptors in spinal dorsal neurons in neuropathic pain. While the mechanisms causing glial GTs in the spinal dorsal horn to be dysfunctional are not clear, activation of glial cells and subsequent release of proinflammatory cytokines have been associated with dysfunctional glutamate uptake in glial cells. For example, studies of the forebrain area showed that tumor necrosis factor-α and interleukin-1β decreased glial GT expression and glutamate uptake in brain slices (Zou and Crews 2005) and cultured hippocampal astrocytes (Ye and Sontheimer 1996). Additionally, downregulation of glial GT protein expression in the spinal dorsal horn induced by nerve injury was reduced by a glial modulator propentofylline (Tawfik et al. 2008). Thus conceivably, glial GTs are bona fide molecules impaired by activation of glial cells and subsequent release of proinflammatory cytokines.

In conclusion, we have identified impaired glial GT as a key mediator used by glial cells to directly cause enhanced activation of sensory neurons in the spinal dorsal horn (central sensitization) (Woolf 1983). Hence, remedying the impaired glial GTs may provide a novel avenue for the treatment of pathological pain.
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Weng HR, Chen HJ, Cata JP. Inhibition of glutamate uptake in the spinal cord induces hyperalgesia and increased responses of spinal dorsal horn neurons to peripheral afferent stimulation. Neuroscience 138: 1351–1360, 2006.


